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HOMOLOGOUS RECOMBINATION IN MISMATCH REPAIR INACTIVATED EUKARYOTIC CELLS

RELATED APPLICATIONS

[0001] The present application is a Continuation-in-Part of United States application Serial No. 09/147,712, filed February 23, 1999, abandoned, which is the National Stage of International application PCT/EP95/02980, filed July 26, 1995.

BACKGROUND OF THE INVENTION

[0002] The introduction of specific modifications in the prokaryotic and eukaryotic genome is a powerful tool in studying gene function both at the level of individual cells and in the context of a complete organism. In addition, modification of specific genes may result in the generation of industrially and medically important organisms, whereas correction of defective alleles in eukaryotic cells may provide a substantial step forward in the development of somatic gene-therapy protocols.

[0003] The method of genetic modification relies on the ability of virtually every cell type to exchange DNA sequences with a high degree of nucleotide sequence similarity by a process which is called homologous recombination (Kucherlapati R. *et al.*, 1988). Briefly, the method of genetic modification involves the generation of a so-called targeting construct, a DNA sequence that is largely identical to the specific chromosomal locus to be modified, but differing from this locus by specific modifications. These modifications can be as small as the deletion, insertion or substitution of a single base-pair, or be as large as the deletion or insertion of ten's of kilobase pairs. On entry of the targeting construct into the cell, exchange of sequences flanking the

modification with their chromosomal counterparts, will result in the introduction of the modification into the recipient chromosome.

[0004] The efficiency of homologous recombination in both prokaryotes and eukaryotes strongly depends on complete sequence identity of exchanging DNA strands (Schen, P. *et al.*, 1986; Nassif, N., *et al.* 1993; Waldman, A.S. *et al.*, 1988; Te Riele, H., 1992). Thus, sequence dissimilarities as small as 0.5% can already strongly impede homologous recombination. In several bacterial species, *Escherichia coli*, *Salmonella typhimurium* and *Streptococcus pneumoniae* as well as in the yeast *Saccharomyces cerevisiae* it has been unequivocally shown that the DNA mismatch repair system is responsible for suppressing recombination between homologous but non-identical DNA sequences (Rayssiguier, C. *et al.*, 1989; Claverys, J.P. *et al.*, 1986; Selva, E. *et al.*, 1995).

Genetic modification of mice

[0005] The introduction of specific genetic alterations into the germ line of mice was made possible by a combination of new techniques in molecular biology and embryology that have become available during the last ten years (Capecchi, M. *et al.* 1989; Frohman, M.A. *et al.*, 1989; Hogan, B. 1994). The method entails the introduction of a planned genetic modification in embryonic stem (ES) cells by homologous recombination. ES cells are originally derived from the inner cell mass of 3.5 day old pre-implantation embryos and can be maintained by *in vitro* culture as immortalized cell lines, retaining the undifferentiated state under appropriate culture conditions. At present, a number of ES cell lines are available. ES cells injected into the blastocoel of 3.5 day blastocyst-stage embryos can efficiently compete with the inner-cell-mass cells of the recipient blastocyst in embryonic development thus generating a chimeric mouse consisting of cells derived from the recipient blastocyst and the injected ES cells. Should the *in vitro* modified ES cells contribute

to the germ line of chimeric animals, the ES cell genome can be transmitted to the next generation resulting with animals that carry the introduced modification in all their cells. Intercrossing of such animals reveals the phenotypic consequences of homozygosity of the modified gene.

[0006] The most commonly introduced genetic modification in ES cells is gene disruption leading to gene inactivation. This allows the study of gene function by analyzing the consequences of the absence of a particular gene in the context of a complete organism. Also, many hereditary human diseases result from homozygosity, *i.e* presence of two defective alleles, of specific genes [e.g., cancer predisposition syndromes like hereditary nonpolyposis colorectal cancer (Modrich, P., 1994), Li Fraumeni syndrome (Malkin *et al.*, 1990), retinoblastoma (Hansen, M.F., 1988)]. The generation of mouse models for such diseases by disruption of the mouse homologues of the genes involved provides an invaluable tool for studying hereditary diseases in an experimental setting.

Gene disruption in ES cells

[0007] The inactivation of a specific gene in ES cells via homologous recombination starts with the preparation of a DNA targeting construct (Thomas, K.R. *et al.*, 1987). Two types of targeting constructs can be used. In a replacement-type vector, a drug-resistance marker gene (conferring to the cell resistance to drugs like G418, Hygromycin B, Puromycin, Histidinol) disrupts a sequence which is homologous to a sequence in or around the target gene in the recipient genome; in an insertion-type vector the marker gene flanks the homologous sequence. The targeting construct is introduced into ES cells by electroporation (or alternatively by Ca-Phosphate precipitation, liposome-mediated DNA transfer or micro-injection) and cells are selected for stable integration of the targeting construct into the recipient genome by growth in medium

containing the appropriate drug. Drug-resistant colonies are the result of either one of two events: integration of the targeting construct at a random site in the genome or integration of the marker gene in the target locus via homologous recombination between the flanking sequences and their chromosomal counterparts. The replacement-type vector integrates by homologous recombination on both sides of the marker gene: the insertion-type vector integrates by a single homologous recombination event leading to duplication of the region of homology. Thus, the marker gene serves two purposes: it allows selection of cells that have taken up the targeting construct and, on integration via homologous recombination, it will disrupt the target gene thereby modifying its function.

[0008] Distinguishing ES cell clones resulting from homologous recombination from those resulting from random integration, requires the DNA of individual clones to be analyzed by Southern hybridization or the polymerase chain reaction.

[0009] Unfortunately, in many targeting experiments, random integration was often found to be far more efficient than homologous recombination and also large variations in targeting efficiency were observed for different genes (Camerini-Otero, R.D. *et al.* 1990). In this respect, mammalian cells differ from bacteria and lower eukaryotes like yeast (Sulston, J.E. *et al.*, 1977), *Leishmania major* (Cruz., A., 1990) or *Trypanosoma brucei* (Ten Asbroek, A. *et al.*, 1990), where integration of exogenous DNA into the recipient genome exclusively or predominantly occurs via homologous recombination. To date, three factors clearly affecting the recovery of homologous recombinants in mammalian cells have been identified. First, the frequency of homologous recombination increases substantially with the total length of the homologous sequences up to 14 kilobase pairs (Deng, C. *et al.*, 1992). Second, the expression level of the marker gene at the target locus affects the frequency of recovery of homologous recombinants: low expression may lead to loss

of homologous recombinants, whereas high expression may allow selection of homologous recombinants at an elevated drug concentration (Hanson, K.D. *et al.*, 1993). Third, sequence dissimilarities between the targeting construct and the chromosomal target locus strongly suppress the efficiency of homologous recombination (Te Riele, H. *et al.*, 1992).

High efficiency targeting with isogenic DNA constructs

[0010] The suppression of homologous recombination in ES cells by small sequence dissimilarities became clear by a gene targeting experiment aimed at disrupting the *Retinoblastoma* gene with a neomycin resistance marker gene. Two targeting constructs were prepared carrying the neomycin resistance marker embedded in 10.5 kb of *Rb* sequence. In one construct the *Rb* sequence was derived from an isogenic mouse strain 129, and was therefore identical to the corresponding chromosomal locus in the ES cells, which were also derived from mouse strain 129. In the other construct, the *Rb* sequence was derived from a non-isogenic mouse strain BALB/c (Te Riele *et al.*, 1992; Table 1). The two constructs contained corresponding *Rb* sequences and were therefore largely similar. However, they differed approximately 0.6% at the nucleotide level (which corresponds to the level of sequence polymorphism found in the human population). Thus, in a stretch of 1687 base pairs that was sequenced, the BALB/c sequence differed from the 129 sequence by 9 base pair substitutions, three small deletions (of 1, 4 and 6 nucleotides) and two polymorphic CA-repeats (see Te Riele *et al.*, 1992). On introduction of these constructs in 129-derived ES cells, homologous recombination at *Rb* with the 129-derived construct was 50-fold more efficient than with the nonisogenic BALB/c-derived construct. To provide additional evidence that the suppression of recombination was solely dependent on the polymorphisms between the endogenous locus and the targeting DNA, the inverse experiment was performed, *i.e.*

targeting of a BALB/c-derived ES cell line with the 129- and BALB/c-derived constructs. This experiment yielded the inverse result, *i.e.* a higher targeting efficiency with the BALB/c-derived construct than with the nonisogenic 129-derived construct.

[0011] With a somewhat different targeting construct, consisting of a hygromycin resistance gene embedded in 17 kb of isogenic Rb DNA, we observed that 80% of all Hygromycin B-resistant colonies resulted from homologous recombination (Te Riele *et al.*, 1992). This demonstrates that, in the presence of perfect homology, also in mammalian cells homologous recombination rather than random integration can be the predominant event.

[0012] Although clearly not all problems of gene targeting have been solved, many genes have now been successfully targeted by the use of isogenic DNA targeting constructs. However, genetic modification of cells derived from an outbred organism can become a difficult endeavor as isogenic targeting constructs are not easily available. In this case, efficient gene targeting would require the targeting construct to be prepared from DNA derived from the target cell. Especially in the context of gene therapy, this would raise a tremendous practical obstacle to correction of a defective gene. Also, base sequence divergence imposes a major barrier to exchanging a large chromosomal region of one species by the syntenic region of another species.

The introduction of subtle mutations

[0013] A related problem exists in methodologies to introduce subtle mutations into the germ line of a transgenic animal. Although protocols for disruption of genes in inbred ES cell lines (and in somatic cell lines of which isogenic DNA targeting constructs can be prepared) are rather well developed, the introduction of more subtle mutations is not straightforward. Current protocols are variations on a two-step procedure

in which first a marker gene is introduced into the target gene followed by replacement of the marker gene by the desired subtle mutation (Te Riele, H. *et al.*, 1992). This procedure requires the marker gene to be selectable both for its presence (first step) and its absence (replacement by the subtle mutation).

[0014] Useful marker genes are the *Hprt* minigene to be used in *Hprt*-deficient ES cells (positive selection in HAT medium: negative selection by 6-thioguanine) and a combination of the neomycin resistance gene (positive selection by G418) and the Herpes Simplex Virus thymidine kinase gene (negative selection by Gancyclovir). In an alternative procedure, the subtle mutation and the marker gene are present on the same targeting construct and concomitantly introduced into the genome by homologous recombination. If an insertion-type vector was used, the marker gene can be removed by intrachromosomal recombination between the duplicated sequences that were generated during the first integration event (Hasty, P. *et al.*, 1993). In case of a replacement-type vector, the marker gene can be removed if it was flanked by two site-specific-recombination sites (e.g. *loxP* sites). Recombination between these sites on introduction into the cell of the *loxP*-specific recombinase *Cre* will remove the marker gene from the genome (Kilby, N. *et al.*, 1993).

[0015] Although either of the above mentioned procedures has allowed the subtle modification of a number of genes in ES cells, they are highly demanding as to the generation of appropriate DNA targeting constructs and the culturing of ES cells under various selective conditions.

[0016] Therefore, an attractive alternative to these procedures might be the use of small single- or double-stranded oligonucleotides (up to 100 bases or base pairs), which are identical to the target locus except for one or several base pair alterations. However, our finding that base sequence dissimilarities as small as 0.6% strongly suppress homologous recombination, may impose a major impediment to using such

oligonucleotides for the introduction of subtle genetic modifications.

There is thus a need in the art to allow the subtle modification of cell lines and cells derived from living organisms and temporarily cultured *in vitro*.

Summary of the Invention

[0017] The present invention provides a method to genetically modify a eukaryotic cell's mismatch repair system in order to allow homologous genetic recombination to occur with non-identical genetic sequences. The present methodology thus overcomes the major barrier that sequence divergence has imposed on prior homologous genetic recombination methodologies. The present invention also can be used to create genetically modified embryonic stem cells which can be used to generate chimeric animals and chimeric plants that can transmit the mutated genome through the germ line. A cell line derived from any mammalian species or mammalian organ, from any organism containing non-identical homologous chromosomes, or from any plant species and cultured *in vitro* can be used for the target cell. This modified target cell can be reintroduced into the organism. The present invention further provides a method to derive cells or cell lines from mismatch repair deficient mice carrying a disruption in both copies of the *Msh2* gene or another mismatch repair gene.

[0018] The invention also provides the introduction of subtle modification of cell lines and cells derived from living organisms and temporarily cultured *in vitro*. Such subtle mutations may be introduced by targeting DNA that can be derived from the same species as the target cell and modified *in vitro* at specific sites to deliberately introduce sequence divergence. Alternatively, the targeting DNA can be derived from the same species as the target cell, where the DNA is non-isogenic with the target cell DNA, and where the two sequences differ up to 5% at the nucleotide level. In another embodiment, the targeting DNA can be

derived from another species as the target cell, and targeting sequence and the target locus can differ by up to 30% sequence divergence in the region where homologous recombination can take place, wherein the targeting DNA may be a chromosomal DNA fragment carried on a YAC or cosmid vector. In yet another embodiment, the targeting DNA is a double- or single-stranded oligonucleotide consisting of 10-100 bases or base pairs of which one or several bases or base pairs differ from the target locus.

[0019] The targeting DNA may be constructed in such a way to allow the generation of deletions of chromosomal regions. A targeting construct thus may consist of any selectable marker gene flanked by two sequences that can combine with chromosomal loci: one flanking sequence being identical or highly homologous (>95% sequence identity) to a sequence of the genome of the target cell, the other flanking sequence being a so-called repetitive sequence. A repetitive sequence may be selected from a sequence having numerous diverged copies spread over the genome, such as a long interspersed element (LINE), a short interspersed element (SINE), *e.g.* an Alu sequence, or a transposon or retroviral sequence. Alternatively, a targeting construct may consist of any selectable marker gene flanked by two sequences, one being a sequence that acts as a telomere, the other flanking sequence being a repetitive sequence having numerous diverged copies are spread over the genome.

Brief Description of the Drawings

[0020] Fig. 1. Targeted disruption of the mouse *Msh2* gene.

[0021] A: Top: schematic diagram of the mouse *Msh2* protein. ATP binding and HTH: putative ATPase and helix turn helix domains, respectively (Valet *et al.*, 1994). The position at which the protein is disrupted by insertion of the Hyg maker is

indicated. Bottom: the genomic *Msh2* locus; indicated below is the targeting construct carrying the *Hyg* maker inserted into a unique *SnaB* I site within an exon sequence of *Msh2*. The positions of the external probes used for detection of targeting events are indicated.

[0022] B: The *Msh2* locus in *Msh2*+/+ cell line wt-2, *Msh2*+/- cell line *sMsh2*-55 and *Msh2*-/- cell line *dMsh2*-9. Genomic DNA of these cell lines was digested with *Eco*RI and analyzed by Southern hybridization using probe 1 (Fig. 1A). Arrowheads indicate the positions of the wild type (wt, 20 kbp), and disrupted (ko, 7.5 kbp) alleles.

[0023] Fig. 2. Mismatch-binding activity in wild-type and *Msh2*-deficient cells.

[0024] Binding activity in extracts of *Msh2*+/+ cell line wt-2 and *Msh2*-/- cell line *dms*h2-9 to double-stranded oligonucleotides containing a mismatch (G.T, G.A, G.G, or A.C), an extrahelical dinucleotide (Extrahelical TG), or an extrahelical 14-nucleotide loop (LOOP 14) and a Homoduplex was assessed using a gel retardation assay. Sequences of the oligonucleotides are given under Experimental Procedures. Arrowheads indicate the positions of mismatch-specific complexes. E2F: Binding to an oligonucleotide carrying an E2F site served as a positive control (Beijersbergen *et al.*, 1995).

[0025] Fig. 3. Microsatellite stability in wild-type and *Msh2*-deficient cells.

[0026] Genomic DNA of 24 subclones derived from *Msh2*+/+ cell line wt-2 and *Msh2*-/- cell line *dms*h2-9 was amplified by PCR using primer sets *D7Mit17* and *D14Mit15*. Arrowheads indicate microsatellite alleles with a detectable length alteration.

[0027] Fig. 4. Tolerance of Msh2-deficient cells to the simple methylating agent MNNG.

[0028] ES cell lines wt-2 (*Msh2*+/+), *sMsh2*-55 (*Msh2*+/-) and *dMsh2*-9 (*Msh2*-/-) were exposed to increasing amounts of MNNG for one hour in the presence of *O*⁶-benzylguanine. After four days of incubation, cells were trypsinized and counted.

Detailed Description of the Preferred Embodiments

[0029] We now have demonstrated that in mammalian cells deficient for the DNA mismatch repair gene *Msh2*, homologous recombination has lost the requirement for complete sequence identity between exchanging DNA sequences. This finding provides a new method for modifying the eukaryotic genome using DNA targeting constructs which substantially differ from the target locus in the region where recombination takes place by genetically and/or functionally inactivating the cell's mismatch correction system.

[0030] Because inactivation of the DNA mismatch repair system eliminates the requirement for DNA targeting constructs that are largely identical to the target locus, efficient genetic modification of both somatic cells and the germ line of outbred organisms is possible. The present technique thus provides a means for somatic gene therapy and the modification of eukaryotic species of which inbred strains are not easily available. Additionally, genomic sequences can be replaced by small oligonucleotides carrying one or more base pair alterations or by large chromosomal sequences derived from other species. Large deletions by intra- or extrachromosomal homologous recombination between repeated but diverged sequences can be created from the use of this method.

[0031] The DNA mismatch repair system can be inactivated (1) by disrupting both copies of one of the genes essential to the mismatch repair system, (2) by introducing antisense RNA constructs, driven by an

appropriate promoter, thus functionally inactivating one of the genes essential for DNA mismatch repair, (3) by introducing a modified antisense oligodeoxynucleotide, thus functionally inactivating one of the genes essential for DNA mismatch repair, (4) by expression of a dominant negatively acting version of a gene involved in DNA mismatch repair, or (5) by saturation of the mismatch repair system through the introduction into the cell of DNA molecules carrying one or more mis- or unpaired bases.

[0032] A gene targeting assay may be used to test whether the cellular mismatch repair system has been inactivated. This test compares the targeting efficiency of isogenic versus nonisogenic DNA targeting constructs or assesses intrachromosomal recombination between homologous, but diverged, DNA sequences. Further, homologous recombination can be tested by using an Embryonic Stem cell line or any other cell line in which the mismatch repair system is inactivated by disruption of one or both copies of the *Msh2* gene or another mismatch repair gene.

[0033] The targeting DNA may be constructed in such a way to allow the generation of deletions of chromosomal regions. A targeting construct thus may consist of any selectable marker gene flanked by two sequences that can combine with chromosomal loci: (1) one flanking sequence being identical or highly homologous (>95% sequence identity) to a sequence of the genome of the target cell, the other flanking sequence being a so-called repetitive sequence, or (2) one being a sequence that acts as a telomere, the other flanking sequence being a repetitive sequence. A repetitive sequence may be selected from a sequence having numerous diverged copies spread over the genome, such as a long interspersed element (LINE), a short interspersed element (SINE), e.g. an Alu sequence, or a transposon or retroviral sequence. Repetitive sequences are described in Watson *et al.*, 1995, for example.

[0034] As described above, base sequence dissimilarities as modest as 0.6% impose a strong barrier to efficient homologous recombination in mouse embryonic stem cells (Te Riele *et al.*, 1992). Suppression of homologous recombination by small base sequence divergences was earlier observed in bacteria (*E. coli*, *S. typhimurium* and *S. pneumoniae*), yeast and mouse fibroblasts (Schen, P., 1986; Nassif, N., 1993; Waldman, A.S., 1988). The role of the DNA mismatch correction system in suppressing recombination between homologous but diverged sequences, was most dramatically demonstrated by Radman and coworkers in studying bacterial conjugation between the related but diverged species *Escherichia coli* and *Salmonella typhimurium* (Rayssiguier, C., 1989). This process relies on entry of chromosomal fragments of one species into the other and recombination of these fragments with the chromosome of the recipient bacterium. The sequence divergence between the two species is estimated to be 20-30% and therefore the recovery of exconjugants from an interspecies cross is about 2×10^5 -fold lower than from an intraspecies cross. However, the recovery of exconjugants from the interspecies cross increased 3×10^3 -fold if the recipient bacteria carried an inactivating mutation in either the *mutS* or *mutL* gene, both of which being essential for DNA mismatch correction.

[0035] The central protein in DNA mismatch correction in *E. coli* is encoded by the *mutS* gene (Modrich, P., 1991). It recognizes and binds to base mispairs and small loops of up to four unpaired nucleotides. After binding to heteroduplex DNA, the MutS-DNA complex is bound by the *mutL* gene product which leads to excision of a tract of single-stranded DNA of up to several kilobases that contains the mispaired nucleotide(s). In this process also the *mutU* gene product plays a role, whereas the *mutH* gene product ensures removal of newly synthesized strands rather than parental strands. The repair process is completed by re-synthesis of

the excised strand and ligation of the remaining nick. Mismatch repair in *E. coli* is responsible for maintaining genome stability in at least two ways:

[0036] i) by recognizing and repairing mis- and unpaired nucleotides that occur, respectively, by misincorporation and slippage during DNA replication;

[0037] ii) by recognizing mismatches occurring in heteroduplexes formed at initial stages of recombination between homologous but not-identical sequences.

[0038] This may either lead to blocking elongation of heteroduplex formation or dissociation of the heteroduplex thus aborting the recombination reaction. Consequently, *E. coli* strains defective for either *mutS* or *mutL* have a pleiotropic phenotype: an increased mutation rate, including destabilization of simple-sequence repeats and an increased rate of recombination between homologous but diverged DNA sequences. The latter phenotype is clearly manifested by the efficient recovery of recombinant bacteria resulting from conjugational crosses between the related but diverged species *Escherichia coli* and *Salmonella typhimurium* wherein the recipient bacterium was deficient for *mutS* or *mutL* (Rayssiguier, C. *et al.*, 1989). Also, the frequency of chromosomal rearrangements by ectopic recombination between diverged sequences is substantially elevated in mismatch repair deficient bacteria (Petit, M.A. *et al.*, 1991).

[0039] In many respects, the biochemistry of mismatch repair systems in eukaryotes resembles that of the *E. coli mutS,L* system. Homologs of both genes have been identified in yeast and mammalian cells. Based on mismatch binding *in vitro*, and on the mutator and recombinator phenotypes of *Saccharomyces cerevisiae* mutants, the protein encoded by the yeast *MSH2* gene seems to be the functional homologue of MutS (Reenan, R.A.G. *et al.*, 1992; Miret, J.J. *et al.*, 1993; Alani, E. *et al.*,

1995). A homolog of the yeast *MSH2* gene was identified in mammalian cells by analysis of a G·T-mismatch-binding activity, positional cloning and PCR amplification of mouse DNA using degenerate primers (Varlet, I. *et al.*, 1994). Similarly, homologues of the *E. coli mutL* gene were identified in yeast and mammalian cells.

[0040] Interestingly, inherited mutations in human *mutS* and *mutL* homologues were recently found to be related to the cancer predisposition syndrome HNPCC (hereditary nonpolyposis colorectal cancer), which is characterized by development of tumors of the proximal colon at early age. In these tumors, mismatch repair is lost, as manifested by destabilization of simple sequence repeats, the replication error-positive (RER⁺) phenotype (Modrich, P., 1994).

Experimental Methods used and Results

[0041] We demonstrate:

- a method of the inactivation of the mismatch repair system by knocking out the function of *msh2* gene;
- the consequences of the mismatch repair inhibition on the phenotype of the embryonic stem cells;
- the effect of inhibiting the mismatch repair system on recombination of diverged sequences; and
- the effect of knockout of the *msh2* genes on phenotype of a transgenic mouse.

Knockout of *msh2* gene in embryonic stem cells

[0042] To demonstrate the role of the mammalian *Msh2* gene in DNA mismatch repair and to address the role of mismatch repair in maintaining genome stability, we generated an ES cell line carrying a disruption in both copies of the mouse *Msh2* gene. This line is designated dMsh2-9.

Disruption of *Msh2* in mouse ES cells

[0043] Genomic *Msh2* fragments were obtained by screening a 1290LA-derived genomic DNA library with a murine *Msh2* cDNA probe (Varlet *et al.*, 1994). The targeting construct was prepared by subcloning a 12.5 kbp BamH I fragment and inserting a hygromycin resistance gene (from PGK*hyg*, Te Riele *et al.*, 1990) into the unique *Sna*B I site located within an exon sequence of *Msh2*. Cloning procedures were performed according to Sambrook *et al.* (1989).

[0044] The targeting construct was separated from vector sequences by gel electrophoresis, purified by electroelution and introduced into 1290LA-derived ES cell line E14 by electroporation as described (Te Riele *et al.*, 1992). Electroporated cells were seeded onto gelatin-coated 10 cm dishes (10^7 cells per plate) and subjected to hygromycin B selection (150 μ g per ml) in BRL-conditioned medium (Hooper *et al.*, 1987) the following day. After 10 days, individual hygromycin B-resistant colonies were randomly picked and expanded on mouse embryonic fibroblasts feeder layers. DNA was extracted from expanded colonies, digested with *Eco*R I and analyzed by Southern hybridization using probes flanking both sides of the targeting construct (Fig. 1A). Two cell lines were obtained out of 135 hygromycin B-resistant colonies showing bands diagnostic for correct integration of the *hyg* marker between codons 588 and 589 of one copy of the *Msh2* gene. These cell lines were designated *sMsh2*-42 and *sMsh2*-55. One cell line obtained from this experiment, designated wt-2, carrying a randomly integrated *hyg* gene, was used as an *Msh2*^{+/+} control.

[0045] To obtain an ES cell line carrying a disruption in both copies of *Msh2*, 10^6 cells of cell line *sMsh2*-55 were plated onto 10 cm plates and cultured in BRL-conditioned medium containing 1.0 or 1.5 mg per ml of hygromycin B. After 12 days of culturing in selective medium and 7 days

in non-selective medium, 24 colonies were obtained which had survived 1.5 mg per ml of hygromycin B. DNA was extracted from expanded colonies, digested with *EcoR* I and analyzed by Southern hybridization. One cell line, designated *dMsh2*-9, carried two disrupted *Msh2* alleles, had lost the wild-type copy and contained the normal number of chromosomes. ES cell lines which had survived 1.0 mg per ml of hygromycin B still contained one disrupted and one wild-type *Msh2* copy. One of these *Msh2*^{+/+} lines was designated *sMsh2*-21 and used for several experiments. Diploidy of cell lines *sMsh2*-55 and *dMsh2*-9 was verified by karyotyping (not shown).

[0046] No *Msh2* transcript could be detected in ES cell line *dMsh2*-9 by Northern blotting (not shown), indicating that the *hyg* marker strongly suppressed *Msh2* expression. The genotype of ES cell line *dMsh2*-9 will therefore be indicated by *Msh2*^{-/-}.

[0047] To verify the absence of mismatch repair due to the knockout of both *msh2* alleles the mismatch binding activity in the mismatch repair deficient *msh-2*^{-/-} and wild-type cells was compared.

[0048] Two different mismatch-binding activities have been described in mammalian cell extracts using gel-retardation assays:

[0049] (i) An activity that recognizes G.T mismatches (Jiricny *et al.*, 1988; Stephenson and Karran, 1989; Griffin and Karran, 1993), an extrahelical T.G dinucleotide (Aquilina *et al.*; 1994) representing an intermediate in replicational slippage of a microsatellite and, weakly, G.A, G.G, A.C. and G.U mismatches (Stephenson and Karran, 1989; Hughes and Jiricny, 1992).

[0050] (ii) In one cell line, an independent activity was detected that recognizes A.C mismatches and also pyrimidine-pyrimidine mismatches (Stephenson and Karran, 1989). Based on protein sequencing, the purified G.T-binding activity was shown to contain MSH2 protein (Palombo, *et al.*, 1994).

[0051] To characterize the mismatch binding properties of the *Msh2*^{-/-} ES cell line, we have performed gel-retardation assays using cell extracts of wild-type ES cell line wt-2 and Msh2-deficient line *dMsh2-9*.

Gel shift assay

[0052] Preparation of cell extracts, annealing of oligonucleotides, binding of cell extracts to duplex oligonucleotides containing mismatched or extrahelical nucleotides, and nondenaturing polyacrylamide gel electrophoresis were performed essentially as described (Stephenson and Karran, 1989). However, gel electrophoresis was performed in TAE buffer rather than in TBE buffer. To obtain duplex oligonucleotides, the oligonucleotide U: 5'-

GGGAAGCTGCCAGGCCCCAGTGTGAGCCTCCTATGCTC-3' (sequences were derived from Aquilina *et al.*, 1994) was radiolabeled and annealed with any of the following unlabeled oligonucleotides: L-G.T:

5'-GAGCATAGGAGGCTGACATTGGGGCCTGGCAGCTTCCC-3' (resulting in a G.T mismatch); L-G.A: 5'-

GAGCATAGGAGGCTGACAATGGGGCCTGGCAGCTTCCCC-3' (resulting in a G.A mismatch); L-G.G: 5'-

GAGCATAGGAGGCTGACAGTGGGGCCTGGCAGCTTCCC-3' (resulting in a G.G mismatch); L-A.C: 5'-

GAGCATAGGAGGCTGACACCGGGCCTGGACAGCTTCCC-3' (resulting in an A.C mismatch); L-TG: 5'-

GAGCATAGGAGGCTGACACTGTGGGGCCTGGCAGCTTCCC-3' (resulting in an extrahelical TG dinucleotide); L-HOM: 5'-

GAGCATAGGAGGCTGACACTGGGGCCTGGCAGCTTCCCC-3' (resulting in a homoduplex); L-LOOP14: 5'-

GAGCATAGGAGGCTGACACATACGTGAGTACTCTGGGGCCTGGCAGCT TCCC-3' (resulting in an IDL loop of 14 extrahelical nucleotides). In all assays, a twofold excess of unlabeled homoduplex competitor oligonucleotide was included. As a positive control, a duplex

oligonucleotide containing the binding site for the E2F family of transcription factors was used (Beijersbergen *et al.*, 1995).

[0053] Results of these experiments as shown in Fig. 2 clearly demonstrate binding activity in wild-type cell extracts to a G.T mismatch and to an extrahelical TG dinucleotide. Binding to both oligonucleotides was entirely absent in the extract of the *Msh2*^{-/-} ES cell line, demonstrating the involvement of the Msh2 protein in this binding activity. As shown before (Hughes and Jiricny, 1992), binding was abolished by the inclusion of ATP in the binding reaction (not shown). We were unable to detect specific binding to G.A., G.G, or A.C mismatches under the conditions used in wild-type or mutant cell extracts. In addition, no Msh-2 dependent binding to a 14-nucleotide IDL was observed (Fig. 2).

Microsatellite formation

[0054] Subclones of ES cell lines *dMsh2*-9 and wt-2 were generated by seeding cells onto mouse embryonic fibroblasts feeder layers at a density of 10³ cells per 10 cm². At that time, the ES cell lines were in culture for approximately 20 divisions since their generation. Twenty-four colonies from each cell line were expanded. Chromosomal DNA was isolated and subjected to the polymerase chain reaction using two end-labeled primer pairs (*D14Mit15* and *D7Mit17*, Dietrich *et al.*, 1994). Amplified products were electrophoresed on a denaturing polyacrylamide gel.

[0055] Whereas none of the wt-2 sublines showed any alteration in microsatellite length, clear length alterations in many *dMsh2*-9 sublines were seen for both microsatellites (8 out of 24 for marker *D7Mit17* and 6 out of 24 for marker *D14Mit15*, Fig. 3). This observation strongly suggests that the Msh2 protein is involved in the repair of slipped replication intermediates. The microsatellites-slippage rate was estimated to be 10⁻² to 10⁻³ per generation.

Mutation Frequency

[0056] The effect of loss of the *Msh2* gene on the mutation frequency of a functional gene was analysed. To this purpose, 6×10^6 cells of ES cell lines *dms2-9* and wt-2 were plated onto 486 cm² gelatin-coated tissue culture surface in BRL-conditioned medium. After two days, 6-Thioguanine (6-TG) was added at a concentration of 10 µg per ml to select for cells that have lost activity of the X-linked *Hprt* gene by mutation. After two weeks the number of resistance colonies was counted.

[0057] Whereas no 6-TG-resistant colonies were seen in the wt-2 culture, 188 resistant colonies were present in the *dMsh2-9* culture. This result extends the mutator phenotype of *Msh2*^{-/-} cells to functional genes.

Tolerance to N-methyl-N¹-nitro-N-nitrosoguanidine (MNNG)

[0058] Mismatch Repair (MMR) was suggested to mediate hypersensitivity to agents that methylate G residues at the O⁶ position. It is believed that MMR recognizes the O⁶-meG-T misrepair that occurs after erroneous incorporation of a thymidine nucleotide opposite to O⁶-meG during replication (Griffin *et al.*, 1994). This is supposedly followed by excision and repair synthesis, again incorporating thymidine opposite to O⁶-meG, triggering a new round of MMR. The net result of this cycling between excision and resynthesis will be the presence of single-stranded regions at the site of O⁶-meG which will lead to double-stranded gaps when replicated during S phase, resulting in cell death (for reviews, see Karran and Bignami, 1992; Karran and Bignami, 1994).

[0059] It is predicted that loss of MMR will present this process, thus conferring tolerance to simple methylating agents. To test this hypothesis directly, survival of *Msh2*^{-/-} cell line *dMsh2-9*, *Msh + 1*- cell line *Msh2-21*

and wild-type cell line wt-2 was determined after exposure to a range of concentration of N-methyl-N¹-nitro-N-nitrosoguanidine (MNNG).

[0060] ES cell lines *dMsh2-9*, *sMsh2-21* and wt-2 were seeded onto MEF feeder layers (mouse embryo fibroblast cells) at a density of 10³ cells per 4 cm³. The following day, cells were exposed for one hour to MNNG ranging in concentration from 0 to 36.45 µM in serum-free medium. After 4 days of incubation, cells were trypsinized, stained with Trypan blue and counted. During the whole procedure from one hour before exposure to MNNG, O⁶-benzylguanine (20 µM) was included in the medium in order to competitively inhibit endogenous methyltransferase activity that might otherwise remove the methyl groups added by MNNG (Dolan *et al.*, 1990).

[0061] Fig. 4 shows that the LD₅₀ for MNNG was increased 20-fold in the *Msh*^{-/-} cell line compared to the heterozygous and wild-type cell lines, directly proving the involvement of Msh2 in determining cellular sensitivity to MNNG. Heterozygous cell line *sMsh2-21* displayed no increased tolerance to MNNG.

[0062] The phenotypic consequences of an *Msh2* deficiency in mouse embryonic stem cells provide clear evidence for an essential role of Msh2 in mammalian DNA mismatch repair. First, *Msh2*-deficient ES cells lack binding activity to a double stranded 38-mer oligonucleotide carrying a G·T mismatch or an unpaired TG dinucleotide. Based on protein sequencing, the purified G.T-binding activity was shown to contain the Msh2 protein (Palombo *et al.*, 1994). To characterize the mismatch binding properties of the *Msh2*^{-/-} ES cell line, we have performed gel-retardation assays using cell extracts of wild-type ES cell line wt-2 and *Msh2*-deficient line *dMsh2-9*. Extracts were incubated with radiolabeled 38-mer double-stranded oligonucleotides containing either a mismatch, an extrahelical dinucleotide, or a 14-nucleotide insertion-deletion-type loop (IDL), followed by electrophoresis on a nondenaturing polyacrylamide gel.

Results of these experiments as shown in Fig. 2 clearly demonstrate binding activity in wild-type cell extracts to a G.T mismatch and to an extrahelical TG dinucleotide. Binding to both oligonucleotides was entirely absent in the extract of the *Msh2*^{-/-} ES cell line, demonstrating the involvement of the *Msh2* protein in this binding activity. As shown before (Hughes and Journey, 1992), binding was abolished by the inclusion of ATP in the binding reaction (not shown). We were unable to detect specific binding to G.A, G.G, or A.C mismatches under the conditions used in wild-type or mutant cell extracts. In addition, no *Msh2*-dependent binding to a 14-nucleotide IDL was observed (Fig. 2).

[0063] Second, *Msh2*-deficient ES cells have a mutator phenotype as evidenced by an at least 150-fold increase in the number of cells resistant to 6-thioguanine, indicating mutational inactivation of the X-linked *Hprt* gene. Moreover, microsatellite length instability was observed in subclones derived from the *Msh2*-deficient ES cell line, but not in subclones derived from wild-type ES cells. Whereas none of the wt-2 sublines showed any alteration in microsatellite length, clear length alterations in many *dMsh2*-9 sublines were seen for both microsatellites (8 out of 24 for marker *D7Mit17* and 6 out of 24 for marker *DI4Mit15*, Fig.3). This observation strongly suggests that the *Msh2* protein is involved in the repair of slipped replication intermediates. The microsatellite-slippage rate was estimated to be 10^{-2} to 10^{-3} per generation. We subsequently investigated the effect of loss of the *Msh2* gene on the mutation frequency of a functional gene. To this purpose, 6×10^6 cells of cell lines wt-2 and *dMsh2*-9 were plated in the presence of 6-thioguanine (6-TG) to select for cells that have lost activity of the X-linked *Hprt* gene by mutation. Whereas no 6-TG-resistant colonies were seen in the wt-2 culture, 188 resistant colonies were present in the *dMsh2*-9 culture. This result extends the mutator phenotype of *Msh2*^{-/-} cells to functional genes.

[0064] Third, *Msh2*-deficient ES cells resisted a 20-fold higher concentration of the methylating agent N-Methyl-N'-Nitro-N-Nitrosoguanidine than wild-type ES cells. MMR was suggested to mediate hypersensitivity to agents that methylate G residues at the O⁶ position. It is believed that MMR recognizes the O⁶-meG.T mispair that occurs after erroneous incorporation of a thymidine nucleotide opposite to O⁶-meG during replication (Griffin *et al.*, 1994). This is supposedly followed by excision and repair synthesis, again incorporating thymidine opposite to O⁶-meG, triggering a new round of MMR. The net result of this cycling between excision and re-synthesis will be the presence of single-stranded regions at the site of O⁶-meG residues which will lead to double-stranded gaps when replicated during S phase, resulting in cell death (for reviews, see Karran and Bignami, 1992; Karran and Bignami, 1994).

[0065] It is predicted that loss of MMR will prevent this process, thus conferring tolerance to simple methylating agents. To test this hypothesis directly, survival of *Msh2*^{-/-} cell line *dMsh2*-9, *Msh*^{+/-} cell line *Msh2*-21 and wild-type cell line wt-2 was determined after exposure to a range of concentrations of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

[0066] In the growth medium, O⁶-benzylguanine was included to competitively inhibit endogenous methyltransferase activity that might otherwise remove the methyl groups added by MNNG (Dolan *et al.*, 1990). Fig. 4 shows that the LD⁵⁰ for MNNG was increased 20-fold in the *Msh2*^{-/-} cell line compared to the heterozygous and wild-type cell lines, directly proving the involvement of *Msh2* in determining cellular sensitivity to MNNG. Heterozygous cell line *sMsh2*-21 displayed no increased tolerance to MNNG.

[0067] Fourth, mice bred to homozygosity for the disrupted *Msh2* allele, originally generated in ES cells, were highly predisposed to tumorigenesis. In *E. coli* (Shen and Huang, 1986), *Drosophila* (Nassif and

Engels, 1993) and mammalian cells (Waldman and Liskay, 1988, Te Riele *et al.*, 1992), the efficiency of recombination between homologous DNA stretches is highly dependent on their sequence identity. We have previously demonstrated that in a gene targeting assay, homologous recombination at the *Retinoblastoma (Rb)* locus in ES cells, derived from mouse strain 129, was 50-fold more efficient with a 129-derived targeting construct than with a construct derived from a non-isogenic (*BALB/c*) mouse strain (Te Riele *et al.*, 1992; Table 1). This construct contained 0.6% base sequence divergence with respect to the isogenic 129 construct. To provide additional evidence that suppression of recombination was solely dependent on the polymorphisms between the endogenous locus and the targeting DNA, we performed the inverse experiment, *i.e.* targeting of a *BALB/c*-derived ES cell line with the 129- and *BALB/c*-derived constructs.

[0068] This experiment yielded the inverse result, *i.e.* a higher targeting efficiency with the *BALB/c* targeting construct than with the non-isogenic 129 targeting construct (Table 1). To investigate whether MMR is responsible for this anti-recombination effect, we have repeated the *Rb* targeting experiment in the (129-derived) ES cell line *dMsh2-9*. We found that in the *Msh2-/-* cell line homologous recombination at the *Rb* locus with the non-isogenic construct was as efficient as with the isogenic construct (Table 1). This experiment demonstrates that *Msh2* is involved in preventing homologous recombination between diverged DNA sequences.

[0069] To study the effect of MMR deficiency on the development of cancer, *Msh2 +/-* ES cell lines *sMsh2-55* and *sMsh2-42* and *Msh2-/-* ES cell line *dMsh2-9* were used to generate chimeric mice. Both the *Msh2* single and double knock-out ES cells gave rise to healthy animals with coat-color chimerism of 20 to 70 %. This result suggests that MMR

deficiency did not alter the capacity of the ES cells to compete with wild-type cells in chimeric development.

[0070] *Msh2* +/- chimeras were found to transmit the mutant allele through the germ line resulting in *Msh2* heterozygous F1 mice. These mice, which may serve as a model for HNPCC, were healthy up to at least 8 months of age (Table 2). Intercrossing of *Msh2* +/- mice resulted in *Msh2* -/- mice according to a normal Mendelian distribution: they were healthy at birth and fertile. However, 30% of the *Msh2* -/- mice developed metastasizing lymphomas of T-cell origin with a peak at 2 months of age (Table 2). In addition, one mouse suffered from generalized histiocytic sarcoma at 3.5 months of age. Extensive histological analyses of these mice did not reveal any other abnormality. Also healthy *Msh2* -/- mice sacrificed at 2 months of age were histologically normal and had normal B- and T-cell populations as evidenced by FACS analysis.

[0071] Accordingly, disruption of *Msh2* leads to inactivation of mammalian DNA mismatch repair.

[0072] By using a gene targeting assay, we demonstrated that it is the mammalian DNA mismatch repair system which is responsible for suppressing homologous recombination between sequences differing as little as 0.6% at the nucleotide level. As described above, homologous recombination at the *Rb* locus with an isogenic DNA targeting construct was 50-fold more efficient than with a similar, but nonisogenic construct containing on the average 0.6% sequence divergence with respect to the target locus (Te Riele, H. *et al.*, 1992). However, in *Msh2*-deficient ES cells, homologous recombination at *Rb* with the nonisogenic targeting construct was as efficient as with the isogenic construct.

Recombination of Diverged Sequences

[0073] In *E. coli* (Shen and Huang, 1986), *Drosophila* (Nassif and Engels, 1993) and mammalian, cells (Waldman and Liskay, 1988, Te Riele *et al.*, 1992), the efficiency of recombination between homologous DNA

stretches is highly dependent on their sequence identity. We have previously demonstrated that in a gene targeting assay, homologous recombination at the *Retinoblastoma* (*Rb*) locus in ES cells, derived from mouse strain 129, was 50-fold more efficient with a 129-derived targeting construct than with a construct derived from a non-isogenic (*BALB/c*) mouse strain (Te Riele *et al.*, 1992; Table 1). This construct contained 0.6% base sequence divergence with respect to the isogenic 129 construct. To provide additional evidence that suppression of recombination was solely dependent on the polymorphisms between the endogenous locus and the targeting DNA the inverse experiment, *i.e.* targeting of a *BALB/c*-derived ES cell line with the 129- and *BALB/c*-derived constructs was performed.

[0074] Targeting and subsequent analysis of the *Rb* locus in a *BALB/c*-derived ES cell line (kindly provided by S. Rastan) and 129/OLA-derived ES cell lines *dMsh2-9* were performed essentially as described (Te Riele *et al.*, 1992). The targeting constructs *129Rb-neo* (derived from the 129OLA genome) and *B/cRb-neo* (derived from the *BALB/c* genome) carry an insertion of the *pMCIneo* marker in exon 19 of the *Rb* gene and differ approximately 0.6% at the nucleotide level (Te Riele *et al.*, 1992).

[0075] Table 1. Homologous recombination has lost dependence on sequence identity in *Msh2* deficient ES cells.

Es cells	Homologous recombination vs total no. G418 ^R col. with	Isogenic vs. non- isogenic	
	<i>129Rb-neo</i>	<i>B/cRb-neo</i>	
<i>BALB/c</i> (<i>Msh2</i> +/+)	1/68 (1.5%)	16/72 (22%)	15 x
129OLA (<i>Msh2</i> +/+)	33/94 (35%)	1/144 (0.7%)	50 x
129OLA (<i>Msh2</i> -/-)	42/185 (23%)	47/184 (26%)	0.9 x

[0076] *129Rb-neo* and *B/cRb-neo* are *Rb* targeting constructs, prepared from the 129 and BALB/c strains of mice, respectively, with 0.6% sequence divergence. Targeting frequencies in 1290LA (*Msh2*+/+) ES cells are derived from Te Riele *et al.*, 1992.

[0077] This experiment yielded a higher targeting efficiency with the BALB/c targeting construct than with the non-isogenic 129 targeting construct. To investigate whether MMR is responsible for this antirecombination effect, we have repeated the *Rb* targeting experiment in the (129-derived) ES cell line *dMsh2-9* (*Msh2*-/-). We found that in the *Msh2*-/- cell line homologous recombination at the *Rb* locus with the non-isogenic construct was as efficient as with the isogenic construct (Table 1). This experiment demonstrates that *Msh2* is involved in preventing homologous recombination between diverged DNA sequences.

[0078] This finding provides a method for modifying the mammalian genome via homologous recombination. By knockout of the *msh2* gene and thus inactivating the mismatch repair system targeted recombination can be achieved despite divergence in the target locus that otherwise prevents recombination in a mismatch repair sufficient background.

Phenotype of *Msh* -/- mice

[0079] Chimeric mice were obtained by injecting 10-15 cells of ES cell lines *sMsh2-55*, *sMsh2-42* and *dMsh2-9* into C57B1/6 blastocysts. Male chimeras obtained with *Msh2* +/- ES cells were crossed with wild-type 1290LA and FYB mice and found to transmit the mutated *Msh2* allele through the germ line. Homozygous *Msh2* mutant mice were obtained by intercrossing F1 heterozygotes. Both the *Msh2* single and double knock-out ES cells gave rise to healthy animals with coat-color chimerism of 20

to 70%. This result evidences that MMR deficiency did not alter the capacity of the ES cells to compete with wild-type cells in chimeric development.

[0080] *Msh2* +/- chimeras were found to transmit the mutant allele through the germ line resulting in *Msh2* heterozygous F1 mice. These mice were healthy up to at least 8 months of age (Table 2).

[0081] Intercrossing of *Msh2* +/- mice resulted in *Msh2* -/- mice according to a normal Mendelian distribution; they were healthy at birth and fertile. However, 30% of the *Msh2* -/- mice developed metastasizing lymphomas of T -cell origin with a peak at 2 months of age (Table 2). In addition, one mouse suffered from generalized histiocytic sarcoma at 3.5 months of age. Extensive histological analyses of these mice did not reveal any other abnormality. Also healthy *Msh2* -/- mice sacrificed at 2 months of age were histologically normal and had normal B- and T -cell populations as evidenced by FACS analysis.

[0082] Table 2. Phenotypes of *Msh2* mutant mice.

Genotype	Age (months)	Condition at birth	Tumor incidence		
			type	frequency**	age (months)
<i>Msh2</i> +/-	8	healthy	lymphoma	0/110	2
<i>Msh2</i> +/+ ::: <i>Msh2</i> -/-*	6	healthy	lymphoma	1/24	2-4
<i>Msh2</i> -/-	4 – 5	healthy	histiocytic sarcoma	6/19	3.5

* Chimeras consisting of wild-type and *Msh2* -/- cells

** Number of mice carrying a tumor per total number in the experiment

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